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Abamectin affects the bioenergetics of liver mitochondria: A potential mechanism of hepatotoxicity

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ABSTRACT

Abamectin (ABA) is a macrocyclic lactone of the avermectin family used worldwide as an antiparasitic agent in farm animals and pets and as the active ingredient of insecticides and nematicides. In this study, the effects of abamectin on the bioenergetics of mitochondria isolated from rat liver were evaluated. Mitochondria are responsible for converting the energy released by electron transport and stored as the binding energy molecule ATP. Xenobiotics that interfere with its synthesis or utilization can be acutely or chronically toxic. Abamectin (5–25 μM) caused concentration-dependent inhibition of the respiratory chain without affecting the membrane potential or the activity of enzymes NADH dehydrogenase or succinate dehydrogenase. This behavior is similar to oligomycin and carboxyatractyloside and suggests direct action on F_oF_1-ATPase and/or the adenine nucleotide translocator (ANT). ABA more pronouncedly inhibited ATPase phosphohydrolase activity in intact, uncoupled mitochondria than in freeze–thawed disrupted mitochondria. ADP-stimulated depolarization of the mitochondrial membrane potential was also inhibited by ABA. Our results indicate that ABA interacts more specifically with the ANT, resulting in functional inhibition of the translocator with consequent impairment of mitochondrial bioenergetics. This effect could be involved in the ABA toxicity to hepatocytes.

1. Introduction

Abamectin (ABA) is obtained by natural fermentation of *Streptomyces avermitilis*, which provides a mixture of avermectins consisting of ⩾80% of avermectin B_{1a} and ⩽20% avermectin B_{1b} (Agarwal, 1998). B_{1a} and B_{1b} (Fig. 1) have similar biological and toxicological properties (Hayes and Laws, 1990). Abamectin is currently used in several countries as a pest control agent in livestock and as an active principle of nematicides and insecticides for agricultural use (Kolar et al., 2008). ABA is highly toxic to insects and may be highly toxic to mammals (Lankas and Gordon, 1989). Seixas et al. (2006) reported that ABA poisoning caused the death of 57 calves over 4 years. The authors noted that this number, caused by incorrect dosage to the animals, might be underestimated because signs of intoxication vary in intensity and many animals recover quickly. Despite its restricted use to animals and crops, several cases of accidental or intentional abamectin poisoning in human also have been described (Chung et al., 1999; Yang, 2008).

Due to its interposition between the digestive tract and the general circulation of the body, the liver has an important role in metabolism and biotransformation of exogenous substances. Therefore, it receives large amounts of nutrients and xenobiotics absorbed through the digestive tract and portal vein, becoming the target organ of several classes of toxicants and natural or synthetic toxins (Guillouzo, 1998). The most direct mechanism of liver toxicity, at the cellular and molecular level, is the specific interaction of the toxicant with a critical cellular component (mitochondria, for example) and subsequent modulation of its function (Meyer and Kulkarni, 2001).

ABA poisoning can impair the function of hepatocytes. Research conducted by Hsu et al. (2001) showed elevated levels of the enzyme aspartate aminotransferase (AST) in the blood serum of rats after exposure to ABA by gavage at doses between 1 and 20 mg/kg body weight. The maximum activity was obtained with a dose of 20 mg/kg of body weight 1 h after ingestion. Eissa and Zidan (2010), using a commercial product, also observed signs of abamectin liver toxicity, with increased activity of the enzyme AST in rats treated with doses equivalent to 1/10 or 1/100 of the LD_{50} (18 mg/kg) in the diet of animals over 30 consecutive days. In addition, El-Shenawy (2010) undertook a comparative study of the *in vitro* toxic action of some insecticides, including ABA at concentrations of 10 and 100 μM, on isolated rat hepatocytes. There was a significant increase in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity when hepatocytes were incubated for 30 min with either concentration of ABA. This activity persisted after 120 min, the longest time point for which data was collected.

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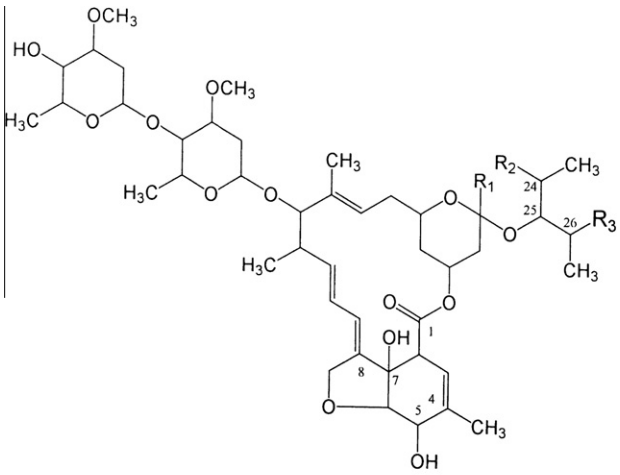

Fig. 1. Chemical structures of abamectin components avermectin B_{1a} and B_{1b}.

Mitochondria carry out a variety of biochemical processes, but their main function is to produce a majority (>90%) of cellular ATP. The proton motive force, whose major impetus is the membrane potential ($\Delta\psi$) generated by electron transport along the respiratory chain in the inner mitochondrial membrane, drives ATP synthesis via oxidative phosphorylation (Mitchell, 1961). Experimental evidence from our research group indicates that mitochondria represent a primary target critical for the action of drugs and toxins (Mingatto et al., 2000, 2007; Garcia et al., 2010). Here, we addressed the actions of ABA on mitochondrial bioenergetics by assessing its effect on respiration, membrane potential, ATP levels, activity of mitochondrial respiratory chain enzymes, ATPase and ANT in isolated rat liver mitochondria.

2. Materials and methods

2.1. Chemicals

Abamectin, containing 92% avermectin B_{1a} and 8% avermectin B_{1b}, was kindly supplied by the company Ourofino Agribusiness (Cravinhos, São Paulo, Brazil). All other reagents were of the highest commercially available grade. Dimethyl sulfoxide (DMSO) used to dissolve abamectin had no effect on the assays. The volume of DMSO added never exceeded 0.1% of the total volume of medium. All stock solutions were prepared using glass-distilled deionized water.

2.2. Animals

Male Wistar rats weighing approximately 200 g were used in this study. The animals, provenient from the Central Bioterium of the São Paulo State University, Botucatu, SP, Brazil, were maintained with a maximum of four rats per cage under standard laboratory conditions, while water and food were provided *ad libitum*. The experimental protocols were approved by the Ethical Committee for the Use of Laboratory Animals of the Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Dracena.

2.3. Isolation of intact and disrupted rat liver mitochondria

Mitochondria were isolated by standard differential centrifugation (Pedersen et al., 1978). Rats were sacrificed by decapitation, and the liver was immediately removed, sliced into 50 ml of medium containing 250 mM sucrose, 1 mM EGTA, and 10 mM HEPES-KOH, pH 7.2, and homogenized three times for 15 s at 1-min intervals with a Potter-Elvehjem homogenizer. Homogenate was centrifuged at 770*g* for 5 min, and the resulting supernatant further centrifuged at 9800*g* for 10 min. The pellet was suspended in 10 ml of medium containing 250 mM sucrose, 0.3 mM EGTA, and 10 mM HEPES-KOH, pH 7.2 and centrifuged at 4500*g* for 15 min. The final mitochondrial pellet was suspended in 1 ml of medium containing 250 mM sucrose and 10 mM HEPES-KOH, pH 7.2 and was used within 3 h. The mitochondrial protein concentration was determined by a biuret assay with BSA as the standard (Cain and Skilleter, 1987).

The disrupted mitochondria were obtained by heat shock treatment after three consecutive cycles of freezing in liquid nitrogen and thawing in a water bath heated to 37 °C. The membrane fragments were kept at 4 °C and were used in the assessment of mitochondrial enzymatic activity within 3 h.

2.4. Mitochondrial respiration assay

Mitochondrial respiration was monitored using a Clark-type oxygen electrode (Strathkelvin Instruments Limited, Glasgow, Scotland, UK), and respiratory parameters were determined according to Chance and Williams (1955). One milligram of mitochondrial protein was added to 1 ml of respiration buffer containing 125 mM sucrose, 65 mM KCl, and 10 mM HEPES-KOH, pH 7.4, plus 0.5 mM EGTA and 10 mM K_2HPO_4, at 30 °C. Oxygen consumption was measured using 5 mM glutamate + 5 mM malate, 5 mM succinate (+2.5 μM rotenone) or 200 μM N,N,N,N-tetramethyl-p-phenylene diamine (TMPD) + 3 mM ascorbate as respiratory substrates in the absence (state-4 respiration) or the presence of 400 nmol ADP (state-3 respiration).

2.5. Estimation of mitochondrial membrane potential ($\Delta\psi$)

The mitochondrial membrane potential ($\Delta\psi$) was estimated spectrofluorimetrically using model RF-5301 PC Shimadzu fluorescence spectrophotometer (Tokyo, Japan) at the 495/586 nm excitation/emission wavelength pair. Safranine O (10 μM) was used as a probe (Zanotti and Azzone, 1980). Mitochondria (2 mg protein) energized with 5 mM glutamate + 5 mM malate were incubated in a medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, pH 7.4, and 0.5 mM EGTA (2 ml final volume).

2.6. ATP quantification

ATP levels were determined using the firefly luciferin–luciferase assay system (Lemasters and Hackenbrock, 1976). After incubation in the presence of ABA, the mitochondrial suspension (1 mg protein/ml) was centrifuged at 9000*g* for 5 min at 4 °C, and the pellet was treated with 1 ml of ice-cold 1 M $HClO_4$. After centrifugation at 14000*g* for 5 min at 4 °C, 100 μl aliquots of the supernatants were neutralized with 5 M KOH, suspended in 100 mM TRIS–HCl, pH 7.8 (1 ml final volume), and centrifuged at 15000*g* for 15 min. The supernatant was worked up with a Sigma/Aldrich assay kit (Catalog Number FLAA) according to the manufacturer's instructions and measured using a SIRIUS Luminometer (Berthold, Pforzheim, Germany).

2.7. Mitochondrial ATPase activity

Mitochondrial ATPase activity was measured in intact-uncoupled and freeze–thawing-disrupted mitochondria according to the protocol of Bracht et al. (2003), with modifications. Intact mitochondria (1 mg protein/ml) were incubated in a medium contain-

ing 125 mM sucrose, 65 mM KCl, and 10 mM HEPES-KOH, pH 7.4, plus 0.2 mM EGTA and 5 mM ATP for 20 min at 37 °C, in the presence of 1 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), in a final volume of 0.5 ml. When disrupted mitochondria were used as the enzyme source, the medium contained 20 mM TRIS–HCl (pH 7.4). The reaction was started by the addition of 5 mM ATP and stopped by the addition of ice-cold 5% trichloroacetic acid. ATPase activity was evaluated by measuring released inorganic phosphate, as described by Fiske and Subbarow (1925), at 700 nm using a DU-800 spectrophotometer (Beckman Coulter, Fullerton, CA).

Results were expressed as nmol Pi. min^{-1}. mg $protein^{-1}$. Sensitivity to oligomycin (1 μg/ml) was tested in all mitochondrial suspensions.

2.8. Determination of enzyme activity related to mitochondrial respiratory chain (NADH and succinate dehydrogenase)

The activity of NADH and succinate dehydrogenases was measured spectrophotometrically according to Bracht et al. (2003), using a DU-800 spectrophotometer (Beckman Coulter, Fullerton, CA). The reaction medium (final volume 1.5 ml) contained 20 mM TRIS, pH 7.4, and 1 μM Antimycin A. Disrupted mitochondria (0.2 mg/ml) were added along with one of four abamectin concentrations (5, 10, 15 and 25 μM), either 1 mM NADH or 10 mM succinate, and 0.4 mM potassium ferricyanide as electron acceptor. The amount of ferricyanide reduced was determined by the decrease in absorbance at 420 nm and enzyme activity was represented as nmol. min^{-1}. mg $protein^{-1}$, using 1.04 mM^{-1} as the molar extinction coefficient of ferricyanide.

2.9. Inhibition of ADP-induced depolarization of $\Delta\psi$

Inhibition of ADP-induced depolarization of $\Delta\psi$ was performed as described (O'Brien et al., 2008) with modifications. Freshly isolated mitochondria were pre-incubated in the presence of 5–25 μM ABA or 5 μM carboxyatractyloside (cATR) and then energized with 5 mM succinate for 1.5 min before adding 400 nmol ADP. ADP-induced depolarization describes the change and recovery in $\Delta\psi$ upon addition of ADP. The amplitude of depolarization induced by ADP was measured in the presence and absence of the test compounds.

2.10. Statistical analysis

Data are expressed as the mean ± S.E. mean, and statistical differences were calculated using one-way analysis of variance (ANOVA) followed by the Dunnetťs test using GraphPad Prism, v 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effects of abamectin on mitochondrial respiration

Mitochondrial oxygen consumption was monitored in the presence of varying concentrations of ABA. The parameters assessed were state-3 respiration (consumption of oxygen in the presence of respiratory substrate and ADP) and state-4 respiration (consumption of oxygen after ADP has been exhausted). At the concentrations tested (5–25 μM), ABA inhibited state-3 respiration of mitochondria in a concentration-dependent manner. This effect was observed when mitochondria were energized with either glutamate plus malate, the respiratory chain site I substrates (Fig. 2A), or succinate, a respiratory chain site II substrate (Fig. 2B). A maximum effect was observed at a concentration of 15 μM. ABA also

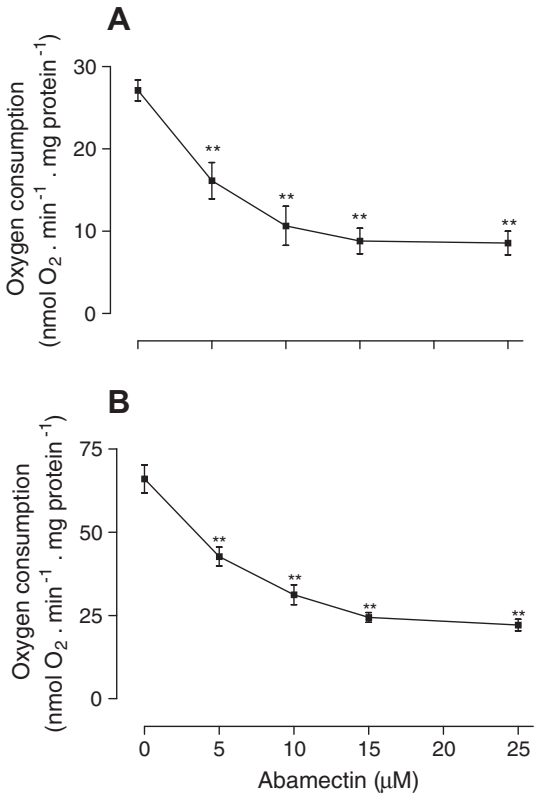

Fig. 2. Effect of abamectin on the state-3 respiration rate of glutamate plus malate (A) and succinate-energized (B) rat liver mitochondria. Assay conditions are described in Section 2. Values represent the mean ± S.E. mean of three experiments with different mitochondrial preparations. **Significantly different from control ($P < 0.01$).

inhibited state-3 respiration of TMPD plus ascorbate-energized mitochondria in a concentration-dependent manner (data not shown). The compound did not stimulate state-4 respiration, indicating that it does not act as an uncoupler (data not shown).

Subsequent experiments with carbonyl cyanide m-chlorophenyl hydrazone (CCCP)-stimulated mitochondrial respiration were performed to test the inhibitor effect of the compound on the respiratory chain or on ATP synthase. ABA did not inhibit CCCP-uncoupled respiration, indicating that only oxidative phosphorylation was inhibited (Fig. 3). The same behavior was ob-

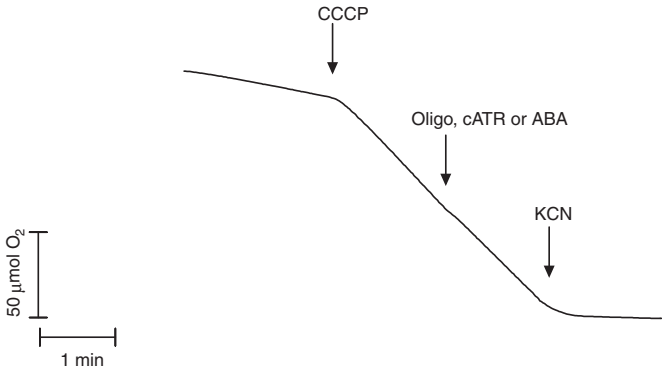

Fig. 3. Effect of abamectin (ABA, 25 μM) on CCCP-uncoupled (1 μM) respiration. The figure is representative of three experiments with different mitochondrial preparations. Arrows indicate addition of compounds. Oligo: oligomycin 1 μg/ml. cATR: carboxyatractyloside 1 μM. KCN: potassium cyanide 1 μM. RFI: relative fluorescence intensity.

served with oligomycin (ATPase inhibitor) and carboxyatractyloside (ANT inhibitor).

3.2. Effect of abamectin on mitochondrial membrane potential ($\Delta\psi$)

Figure 4 shows the effect of ABA on the $\Delta\psi$ of glutamate + malate-energized rat liver mitochondria. ABA (25 μM) did not dissipate $\Delta\psi$. The same behavior was observed for oligomycin and carboxyatractyloside. At the end of the experiment, 1 μM CCCP (uncoupler) or 2.5 μM rotenone (complex I inhibitor) was added as a positive control, and the mitochondrial membrane electrical potential dissipated.

3.3. Effect of abamectin on mitochondrial ATP levels

The effect of ABA on mitochondrial ATP levels was evaluated using the respiratory assay conditions 15 min after mitochondria were incubated with the compound (Fig. 5). In agreement with the mitochondrial respiration results, ABA caused a significant concentration-dependent decrease in mitochondrial ATP levels, reaching a maximum effect at 15 μM.

3.4. Effects of abamectin on the F_oF_1-ATPase activity

The effects of ABA on F_oF_1-ATPase activity were measured in intact-uncoupled mitochondria in the presence of CCCP, and in freeze–thawing-disrupted mitochondria, as shown in Fig. 6A and B, respectively. The ATPase activity of uncoupled mitochondria was increased in a concentration-dependent manner by ABA (Fig. 6A). In disrupted mitochondria, the effects were less dramatic and similar across all concentrations tested (Fig. 6B).

3.5. Effect of abamectin on NADH and succinate dehydrogenase activities

The effect of ABA on NADH and succinate dehydrogenase activity was measured in freeze–thawing-disrupted mitochondria. As expected, ABA at concentrations from 5 to 25 μM did not cause significant changes in enzyme activity (data not shown).

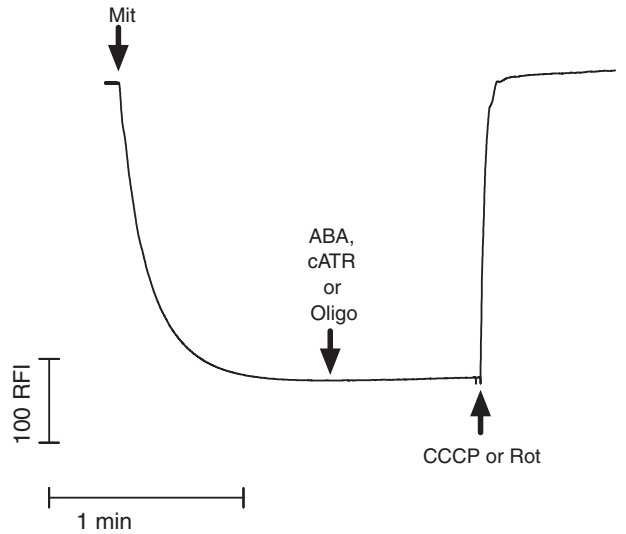

Fig. 4. Effect of abamectin (ABA, 25 μM) on the membrane potential of glutamate plus malate-energized rat liver mitochondria. Assay conditions are described in Section 2. The figure is representative of three experiments with different mitochondrial preparations. Arrows indicate addition of compounds. Mit: mitochondrial suspension 1 mg/ml. Oligo: oligomycin 1 μg/ml. cATR: carboxyatractyloside 1 μM. CCCP: CCCP 1 μM. Rot: rotenone 2.5 μM. RFI: relative fluorescence intensity.

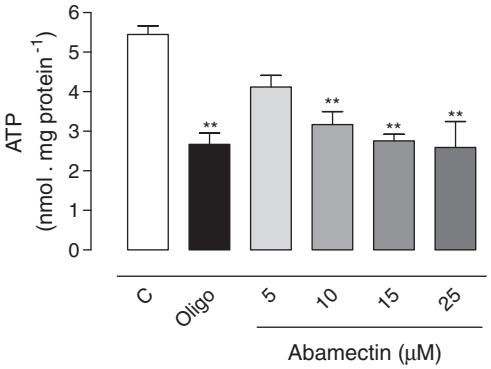

Fig. 5. Effect of abamectin (ABA) on the ATP levels in glutamate plus malate-energized rat liver mitochondria. Assay conditions are described in Section 2. Values represent the mean ± S.E. mean of three experiments with different mitochondrial preparations. C: control, only 0.1% DMSO. Oligo: oligomycin 1 μg/ml. **Significantly different from control ($P < 0.01$).

3.6. Effect of abamectin on ADP-induced depolarization of $\Delta\psi$

The purpose of this assay was to determine whether ABA inhibits ADP-induced depolarization of $\Delta\psi$ by interference with ANT. Carboxyatractyloside was used as a positive control for direct ANT inhibition. ABA caused significant, concentration-dependent inhibition of ADP-stimulated depolarization of $\Delta\psi$ (Fig. 7).

4. Discussion

Mitochondrial dysfunction is a fundamental pathogenic mechanism that leads to several significant toxicities in mammals, espe-

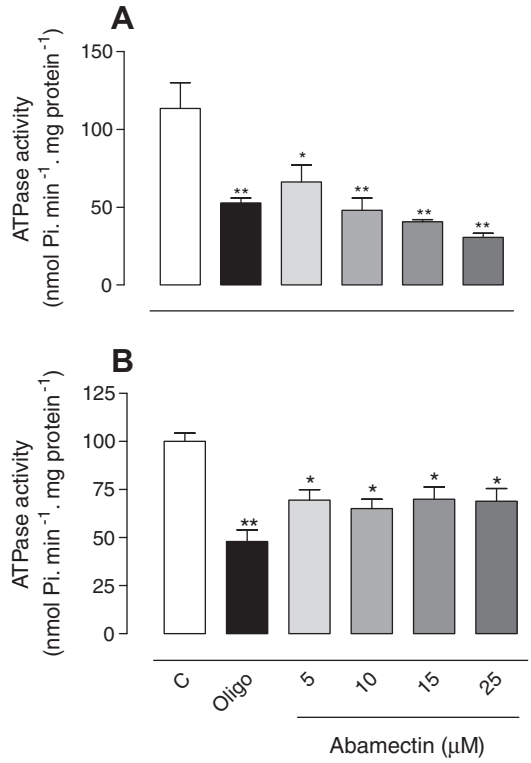

Fig. 6. Effects of abamectin (ABA) on ATPase activity in intact-uncoupled mitochondria in the presence of CCCP (A) and in freeze–thawing-disrupted rat liver mitochondria (B). Assay conditions are described in Section 2. Values represent the mean ± S.E. mean of three experiments with different mitochondrial preparations. C: control, only 0.1% DMSO. Oligo: oligomycin 1 μg/ml. *,**Significantly different from control ($^*P < 0.05$ and $^{**}P < 0.01$).

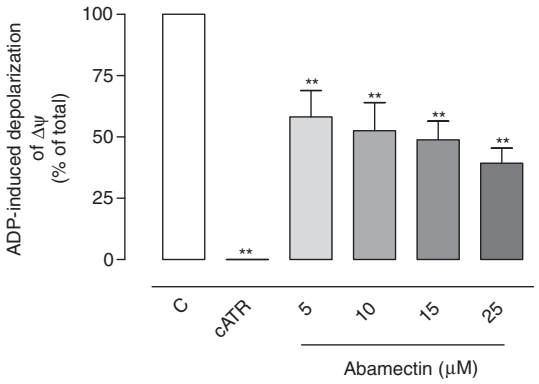

Fig. 7. Effect of abamectin (ABA) on ADP-induced depolarization of $\Delta\psi$. Assay conditions are described in Section 2. Values represent the mean ± S.E. mean of three experiments with different mitochondrial preparations. C: control, only 0.1% DMSO. cATR: carboxyatractyloside 5 μM. **Significantly different from control ($P < 0.01$).

cially those associated with the liver (Szewczyk and Wojtczak, 2002; Amacher, 2005). To assess the potential involvement of mitochondria in ABA-related hepatotoxicity, we assessed its effects on the bioenergetics of rat liver mitochondria. The results obtained using mitochondria energized with glutamate + malate (electron donors to complex I), succinate (electron donor to complex II) and TMPD/ascorbate (artificial donor of electrons to complex IV) showed that ABA inhibits state-3 respiration in a concentration-dependent manner at concentrations from 5 to 25 μM. According to Chance and Williams (1955), state-3 respiration involves mitochondria, ADP and a respiratory substrate, and the speed of ADP phosphorylation is the limiting factor of the process. The inhibition observed in the three experiments may result from the direct action of abamectin on the respiratory chain, or from an inhibitory effect on F_oF_1-ATPase or ANT.

It is possible to distinguish between inhibition of oxidative phosphorylation and inhibition of the electron transport chain by using an uncoupler-stimulated respiration test. If inhibition occurs in electron transport chain, uncoupler-stimulated oxygen consumption will be inhibited. If the tested compound instead acts on the oxidative phosphorylation, it will be innocuous. We conducted such a test using CCCP as an uncoupler and succinate as the substrate. Mitochondrial oxygen consumption was not inhibited by ABA but was inhibited for KCN (respiratory chain complex IV inhibitor), indicating that the inhibition of state-3 respiration by the compound does not occur through direct action on the respiratory chain. The effect is probably due to interaction with F_oF_1-ATPase and/or the ADP/ATP translocator because it is similar to those of oligomycin, a specific inhibitor of F_oF_1-ATPase, and carboxyatractyloside, an ANT inhibitor. In addition, mitochondrial oxygen consumption inhibited by 25 μM ABA was further stimulated with 1 μM CCCP, demonstrating that the mitochondrial respiratory chain was not inhibited (data not shown).

The complex I (NADH dehydrogenase) is the most vulnerable complex of the electron transport chain. The smaller, simpler complex II contains succinate dehydrogenase, the only enzyme of the Krebs cycle linked to the inner mitochondrial membrane (Boelsterli, 2007). We corroborated our results cited in the item 3.5 that saw no ABA effect on NADH dehydrogenase and succinate dehydrogenase.

ABA did not dissipate membrane potential, as do inhibitors of respiratory chain complexes, such as rotenone and uncoupling substances such as CCCP, i.e., those capable of acting on the linkage between ATP synthesis and electron transport. Our results support the hypothesis, proposed earlier, that ABA behaves similarly to oligomycin and/or carboxyatractyloside, indicating that the toxic mechanism of ABA involves direct action on F_oF_1-ATPase and/or ANT.

Because ATP is an essential metabolic component, interference with its synthesis or use is the mechanism by which many xenobiotics express acute or chronic toxicity (Meyer and Kulkarni, 2001). ABA significantly inhibited the synthesis of ATP at 10 μM and reached a maximum effect at 15 μM.

The ANT is an important component of the mitochondrial machinery of ATP synthesis because of its intrinsic adenine nucleotide translocase activity. ANT participates in both pathological (mitochondrial permeability transition formation/regulation and cell death) and physiological (adenine nucleotide exchange) mitochondrial events, making it a prime target for drug-induced toxicity (Oliveira and Wallace, 2006). To demonstrate ABA-induced inhibition of ATPase and/or ANT, we evaluated its effects in the activity of ATPase using intact-uncoupled and freeze–thawing-disrupted mitochondria with an excess of ATP, a condition that drives the enzyme to operate in the reverse direction, hydrolyzing ATP (Bracht et al., 2003), and also in the ADP-induced depolarization of $\Delta\psi$. We saw more significant stimulation of ATPase activity in intact-uncoupled mitochondria than in disrupted mitochondria, which taken together with the observed inhibition of ADP-induced depolarization of $\Delta\psi$ indicates that abamectin more specifically inhibits ANT than F_oF_1-ATPase.

In conclusion, the present study shows that ABA perturbs the mitochondrial bioenergetics through different mechanisms and that its effect on the adenine nucleotide translocator (ANT) is more potent than on F_oF_1-ATPase. These effects constitute a potential mechanism for ABA toxicity in liver cells, which could contribute to the toxicological effects of ABA described in animals and human.

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

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